Uranyl-cytochrome b_5 interaction regulated by surface mutations and cytochrome c*

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Understanding uranium-protein interaction is important for revealing the mechanism of uranyl ion (UO_2^{2+}) toxicity. In this study, we investigated the interaction between UO_2^{2+} and a quadruple mutant of cytochrome b_5 (E44/48/56A/D60A cyt b_5, namely 4A cyt b_5) by spectroscopic approaches. The four mutated negatively-charged surface residues of cyt b_5 have been considered to be the interactive sites with cytochrome c (cyt c). Also, we studied the interaction between UO_2^{2+} and the protein-protein complex of 4A cyt b_5-cyt c. The results were compared to the interaction between UO_2^{2+} and cyt b_5, and the interaction between cyt c and cyt b_5-cyt c complex, from previous studies. It was found that the interaction of UO_2^{2+}-cyt b_5, i.e., uranyl ion binding to cyt b_5 surface at Glu37 and Glu43 as previously proposed by molecular modeling, is regulated by both surface mutations of cyt b_5 and its interacting protein partner cyt c. These provide valuable information on metal-protein-protein interactions and clues for understanding the mechanism of uranyl toxicity.

Keywords: Uranium, Cytochrome, Heme protein, Protein-protein interaction, Fluorescence

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I. INTRODUCTION

Developing nuclear energy is of social and economic importance, while this arises a major concern over environmental pollution by long-lived radioactive wastes, such as uranyl ion (UO_2^{2+}), the most stable form of uranium under physiological conditions [1]. In addition to its radiation hazard, UO_2^{2+} is of high toxicity because it interacts with both DNA [2] and proteins [3–6] and disrupts their biological functions. To date, plentiful proteins have been found to be the targets of UO_2^{2+}, such as transferrin, ferritin and albumin [3–6]. A protein data bank (PDB) survey shows that UO_2^{2+} binds to proteins mainly through carboxylic acid groups such as those of aspartate (Asp) and glutamate (Glu), as well as other coordinating amino acids such as histidine (His) and tyrosine (Tyr) [7]. In previous papers [8, 9], we studied the structural and functional consequences of UO_2^{2+} binding to cytochrome b_5 (cyt b_5), cytochrome c (cyt c), and the protein-protein complex of cyt b_5-cyt c, by both experimental and theoretical approaches.

Cyt b_5 is a small membrane heme protein, which is characterized by a highly negatively charged surface in presence of a series of acidic residues surrounding the heme group, known as an acidic cluster (Fig. 1) [10]. It has been shown that the acidic cluster participates in formation of cyt b_5-cyt c complex [11–17], and is crucial in mediating cyt c signaling in apoptosis [18]. With a positive charge, UO_2^{2+} has a strong tendency to be absorbed at negatively charged surface of membrane [19], where it has a large possibility of interacting with membrane proteins such as cyt b_5. In previous study [8], we proposed a UO_2^{2+} binding site for cyt b_5 at surface residues of Glu37 and Glu43 (Fig. 1), based on molecular modeling and dynamics simulation.

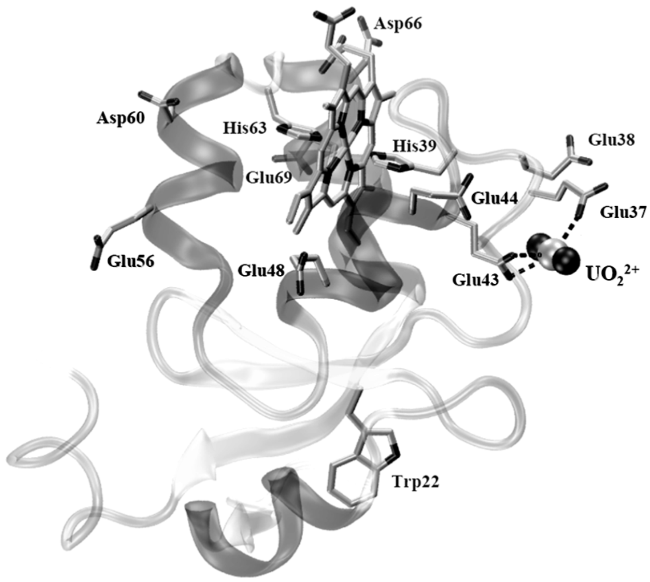

Fig. 1. Modeling structure of UO_2^{2+}-cyt b_5 complex, showing the UO_2^{2+} ion binding to Glu37 and Glu43, the residues in acidic cluster, the heme group and Trp22 in the hydrophobic pocket.

To further probe the role of acidic cluster in uranyl-cyt b_5 interaction and to reveal the effect of surface mutations of cyt b_5 on UO_2^{2+} binding, we herein choose a quadruple mutant of cyt b_5 as a target, in which four acidic residues were replaced with alanine, E44/48/56A/D60A cyt b_5, namely 4A cyt b_5. This mutant was designed previously for studying the interface between cyt b_5 and cyt c, where the replaced residues were considered to be interactive sites [16]. It keeps the uranyl-binding site of Glu37 and Glu43 but has a low binding affinity for cyt c, and therefore is an ideal model protein for investigating the regulation effect of cyt c by a comparison

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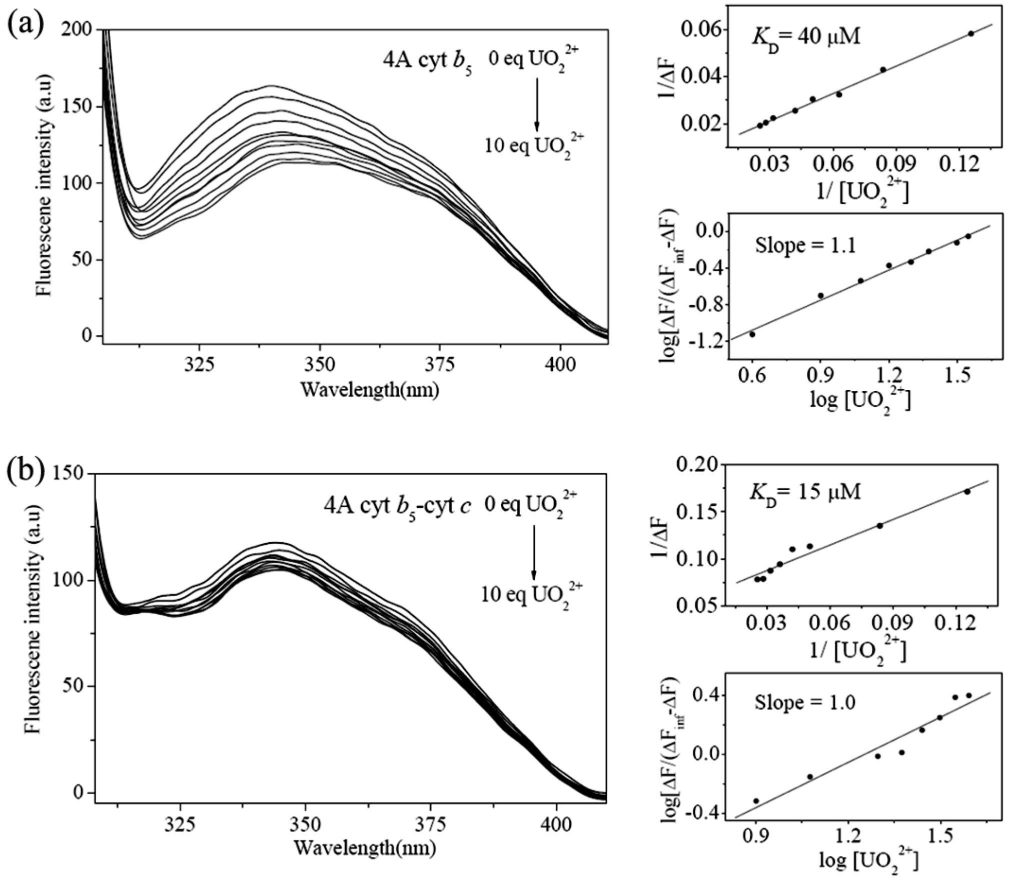

Fig. 2. Fluorescence titration of 4A cyt b_5 (4 μM) (a) and 4A cyt b_5-cyt c complex (4 μM) (b), with UO_2^{2+} in 50 mM Tris·HCl buffer (pH 7.0) at 25 °C. Double reciprocal plot and Hill plot are shown in right top and right down, respectively.

with that of cyt b_5-cyt c complex.

II. MATERIALS AND METHODS

4A cyt b_5 was expressed and purified as described previously [16]. Horse heart cyt c (Type VI) was purchased from Sigma Chemical Co. Uranyl nitrate and other chemicals were commercial products and of analytical grade. Double distilled water was used throughout the experiments.

Fluorescence spectra of 4A cyt b_5 and 4A cyt b_5-cyt c complex (4 μM in 50 mM Tris·HCl buffer, pH 7.0) with titration of UO_2^{2+} up to 10 equivalents, were collected at 25 °C on a LS45 fluorescence spectrometer (Perkin Elmer, USA). The excitation wavelength was 295 nm and emission spectra were recorded from 310 nm to 410 nm. The apparent dissociation constant (K_D) was calculated from double reciprocal plot by fitting to Eq. (1) [20],

$$1/\Delta F = (K_D/\Delta F_{inf})(1/[UO_2^{2+}]) + 1/\Delta F_{inf}, \qquad (1)$$

where ΔF is the difference between the maximum fluorescence in the absence and presence of UO_2^{2+} and ΔF_{inf} is the fluorescence change for the complete binding of UO_2^{2+}. The number of UO_2^{2+} binding site was calculated from ΔF of fluorescence spectra using the Hill plot [21], i.e. the yielded slope.

$$\log[\Delta F/(\Delta F_{inf} - \Delta F)] = slope \log[UO_2^{2+}] \qquad (2)$$

Circular dichroism (CD) spectra of 4A cyt b_5 and 4A cyt b_5-cyt c (20 μM in 50 mM Tris·HCl buffer, pH 7.0), in the absence or presence of 5 equivalents of UO_2^{2+}, were collected at 25 °C from 300 nm to 600 nm (1.0 cm path length), with a Jasco J720 spectrometer (Japan). Dilution effect of addition of UO_2^{2+} was corrected by adding the same volume of buffer solution into the protein solution.

III. RESULTS AND DISCUSSION

The single tryptophan residue (Trp22) in cyt b_5 (Fig. 1) serves as a convenient and sensitive reporter for studying the interaction of UO_2^{2+}-cyt b_5 by fluorescence spectroscopy. Figure 2(a) shows that the fluorescence intensity of 4A cyt b_5 decreases gradually upon titration of UO_2^{2+} ions, suggesting a fluorescence quenching as observed for uranyl titration of transferrin, ferritin and albumin [3–6]. This observation is similar to that of wild-type (WT) cyt b_5 [9]. When the changes of the maximum fluorescence were fitted to a double

reciprocal plot (Fig. 2(a), right up) and a Hill plot (Fig. 2(a), right down), its binding affinity (K_D = 40 μM) is 4 times lower than that of cyt b_5 (Table 1), though 4A cyt b_5 remains a single binding site of UO_2^{2+} (slope = 1.1). It is likely due to a conformational change of residues Glu37 and Glu43 as a result of the four mutations in the acidic cluster, especially for Glu44 close to the proposed uranyl-binding site, as observed in the crystal structure of 4A cyt b_5 (PDB entry 1M2M) [16]. These observations indicate that the negative charges on the acidic cluster of cyt b_5 play important roles in uranyl-protein interaction.

TABLE 1. Binding affinity constants (K_D) for uranyl binding to cyt b_5, 4A cyt b_5, cyt c and the complexes

Uranyl-complex	K_D (μM)	Refs.
UO_2^{2+}-cyt b_5	10	[9]
UO_2^{2+}-4A cyt b_5	40	This work
UO_2^{2+}-cyt c	87	[9]
UO_2^{2+}-cyt b_5-cyt c	30	[9]
UO_2^{2+}-4A cyt b_5-cyt c	15	This work

On the other hand, in titration of 4A cyt b_5-cyt c complex with UO_2^{2+} (Fig. 2(b)), the single uranyl-binding site on 4A cyt b_5 surface in the complex has a higher affinity of UO_2^{2+} (K_D = 15 μM) than that on the surface of isolated 4A cyt b_5 (K_D = 40 μM). This observation suggests that the conformation of surface residues, Glu37 and Glu43, are tuned to be suitable for uranyl-binding as a result of cyt c interacting with 4A cyt b_5, though the binding constant decreases from cyt b_5-cyt c ($2.2 \times 10^4\ M^{-1}$) to 4 A cyt b_5-cyt c ($5.5 \times 10^3\ M^{-1}$), as determined by NMR technique in a previous study [15].

In Table 1, the uranyl-binding factor (K_D =15 μM) of 4A cyt b_5-cyt c complex is two times lower than that of cyt b_5-cyt c complex (30 μM), suggesting that a different protein-protein interface was adopted for 4 A cyt b_5-cyt c complex as a result of mutation of four acidic residues on the same heme-exposed edge side of cyt b_5 (Fig. 1). In an earlier study, Huang and co-workers [17] showed that the charge neutralization of 4 A cyt b_5 greatly increased the relative contribution of the heme propionate and other charged residues surrounding the heme edge to contact with cyt c. Thus, our observations indicate that UO_2^{2+}-cyt b_5 interaction is regulated by both surface mutations of cyt b_5 and its partner cyt c.

The structural perturbations of 4A cyt b_5 and its complex with cyt c upon uranyl binding were studied by circular dichroism (CD) spectroscopy (Fig. 3). It shows that UO_2^{2+}-4A cyt b_5 complex has a slightly decreased negative Cotton effect at 419 nm, whereas the Cotton effect in 300–350 nm is more positive than that of 4A cyt b_5 (Fig. 3(a)), which indicates that, similar to the observation for UO_2^{2+}-cyt b_5 [9], UO_2^{2+} binding to the surface of 4A cyt b_5 slightly alters the conformation of heme-binding domain and aromatic amino acids such as Phe35 and Trp22 in the protein hydrophobic core. The CD spectra also show that upon uranyl binding, the 4A cyt b_5-cyt c complex has an altered positive Cotton effect and a decreased negative Cotton effect compared to that in the absence of UO_2^{2+} ions, with decreased intensity in 300–350 nm (Fig. 3(b)). These spectral changes are similar to the case of UO_2^{2+} binding to cyt b_5-cyt c complex, whereas both cases are less obvious than that for UO_2^{2+} binding to cyt c [9]. These observations suggest that UO_2^{2+} ions alter the heme active site in both 4A cyt b_5-cyt c and cyt b_5-cyt c complexes, which are regulated by cyt c, likely through forming dynamic protein-protein complexes and competing with UO_2^{2+} ions.

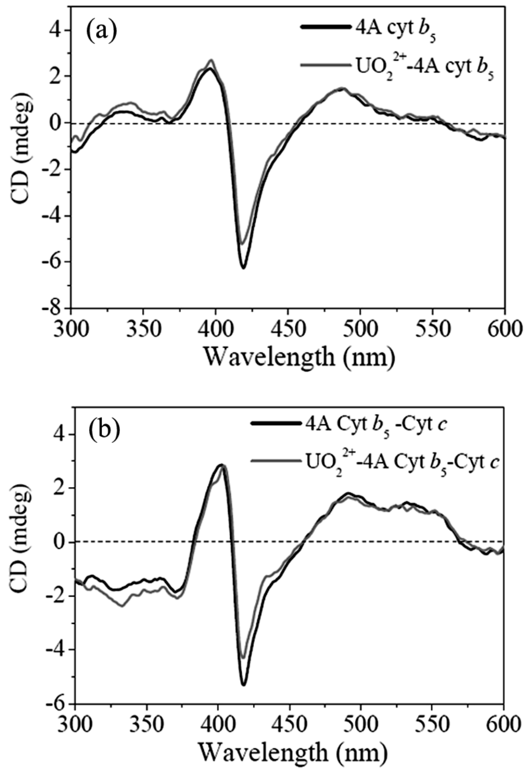

Fig. 3. CD spectra of 4A cyt b_5 (20 μM) (a) and 4A cyt b_5-cyt c complex (20 μM) (b) in the absence (black lines) and presence (grey lines) of UO_2^{2+} (100 μM) in 50 mM Tris · HCl buffer (pH 7.0) at 25 °C.

IV. CONCLUSION

In summary, we investigated the interactions of UO_2^{2+}-4A cyt b_5 and UO_2^{2+}-4A cyt b_5-cyt c complex, and compared to that of UO_2^{2+}-cyt b_5, UO_2^{2+}-cyt c and UO_2^{2+}-cyt b_5-cyt c from our previous studies [8, 9]. With four acidic residues on protein surface replaced by alanine, 4A cyt b_5 exhibits a lower affinity for UO_2^{2+} compared to that for WT cyt b_5, though it remains the proposed uranyl-binding site at Glu37 and Glu43, which suggests a key role of the acidic cluster of cyt b_5 in tuning uranyl-protein interaction. On the other hand, UO_2^{2+} binds more tightly to 4A cyt b_5-cyt c complex than to the cyt b_5-cyt c complex, presumably due to the conformational changes of Glu37 and Glu43 by a protein-protein interface different from that of cyt b_5-cyt c complex. These observations indicate that UO_2^{2+}-cyt b_5 interaction is regulated by both the surface acidic cluster of cyt b_5 and its interacting protein partner cyt c, which

provides valuable information on metal-protein-protein interactions, and clues for understanding the mechanism of uranyl toxicity.

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